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N. Lukianova¹, **T. Zadvornyi**¹, **T. Borikun**¹, **O. Mushii**¹, **A. Pavlova**¹,
A. Tymoshenko¹, **E. Stakhovskiyi**², **I. Vitruk**², **V. Chekhun**¹

¹ R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology,
National Academy of Sciences of Ukraine, Kyiv, Ukraine

² The State Non-Profit Enterprise "National Cancer Institute"
of the Ministry of Health of Ukraine, Kyiv, Ukraine

* Correspondence: Email: nataluk10@gmail.com

SIGNIFICANCE OF OSTEOPONTIN FOR PREDICTING AGGRESSIVENESS OF PROSTATE CANCER

Background. Effective prediction of the course of prostate cancer (PCa) and the stratification of treatment tactics largely depend on the use of prognostic markers that reflect the molecular and biological features of tumors. In view of the important role of matricellular proteins in the modulation of the growing tumor and metastasis of the hormone-dependent neoplasms, **the aim** of the work was to study the expression of osteopontin (OPN) at the protein and mRNA levels in the PCa tissue in order to assess the significance of this protein for predicting the aggressiveness of PCa. **Materials and Methods.** The work is based on the analysis of the results of the examination and treatment of 83 patients with PCa of stages II–IV. The study of OPN expression at the level of mRNA and protein in the PCa tissue was carried out using methods of the real time polymerase chain reaction and immunohistochemistry, respectively. **Results.** The OPN expression in the PCa tissue was 1.6 times ($p < 0.05$) higher in patients with regional lymph node metastases compared to patients without metastases. In patients with a Gleason score of < 7 , the OPN expression in the tumor tissue was 1.4 times lower ($p < 0.05$) than in patients with poorly differentiated PCa. In patients with a high risk of tumor progression, the OPN expression level was 1.4 and 2.1 times higher ($p < 0.05$) compared to patients with a moderate and low risk of PCa progression. The patients with a high OPN expression level in the PCa tissue had significantly decreased 2-year recurrence-free survival rate (by 25%). **Conclusions.** The obtained results indicate the expediency of using OPN expression indicators in the tumor tissue to predict the PCa aggressiveness and assess the risk of its recurrence.

Keywords: prostate cancer, osteopontin, prognosis, survival.

The introduction of prostate-specific antigen (PSA) into routine practice more than three decades ago made it possible to significantly im-

prove the diagnosis of prostate cancer (PCa) and led to a significant increase in the survival rates of patients [1]. At the same time, clinicians faced

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other problems, such as the relatively low specificity of PSA (in 20%—40% of patients with PCa, its levels correspond to the reference values) and overdiagnosis of PCa (detection of a large number of clinically insignificant cases of PCa). In addition, the use of PSA or its derivatives does not allow distinguishing patients with aggressive PCa forms, as well as predicting the course of the disease [2, 3]. For this purpose, a number of clinicopathological indicators are used, such as the stage of the tumor process according to the TNM system, Gleason histological grading scale, as well as the preoperative PSA level in blood serum. However, their application is far from perfect and often leads to an incorrect determination of the degree of malignancy, which complicates the choice of the adequate therapy and is reflected in the long-term outcomes of the PCa treatment [4].

The development of modern technologies during the last two decades has contributed to the discovery of the molecular pathogenesis of PCa and allowed the identification of a wide range of potential markers (p53, Bcl-2, p16INK4A, p27Kip1, c-Myc, AR, E-cadherin, VEGF, etc.) [5]. The created panels of diagnostic and prognostic biomarkers and tests developed on their basis (4K, Phi, Progenesa, T2-ERG, ExoDx, SelectMDx, ConfirmMDx, Prolaris, Oncoytype DX, Decipher) have opened up new opportunities for optimizing early diagnosis and predicting the PCa course, but due to low sensitivity and specificity, none of them found widespread use in clinical practice [6]. This situation is due to the high level of PCa heterogeneity [7], which indicates the urgency of searching for the additional molecular and biological signs associated with cancer aggressiveness.

PCa is often diagnosed in the late stages, which are characterized by the presence of distant metastases, in particular in the bones. With this in mind, researchers focus on studying the mechanisms of metastasis and identifying the main players at the molecular and cellular levels. In

particular, the important role of the RANKL/RANK/OPG system, which is involved in the bone tissue remodeling, has been proven, and the disruption of its homeostasis is one of the key factors in the metastasis of PCa [8]. The data of the recent studies indicate the important role of osteopontin (OPN) or secretory phosphoprotein 1, encoded by the *SPP1* gene, as one of the main proteins of the bone matrix involved in metastasis [9]. The molecular mechanisms that determine the role of OPN in metastasis have not been fully elucidated. OPN plays a role in cell adhesion, chemotaxis, macrophage-directed inhibition of interleukin-10, stress-dependent angiogenesis, prevention of apoptosis, and regulation of the interaction of the cell matrix and cell signaling through binding to integrin and CD44 receptors.

According to the data of several studies [10, 11], the changes in the functional properties of OPN lead to the stimulation of the growth of the malignantly transformed cells, which contributes to the PCa progression. In the previous studies in an *in vitro* system, we established a relationship between OPN expression indicators at the protein and mRNA levels and the degree of malignancy of PCa cells [12, 13]. According to Tilli et al. [14], OPNb and OPNc isoforms are able to stimulate the proliferation and invasion of PCa cells through PI3K-mediated signaling, as well as regulation of the expression of MMP-2, MMP-9, and VEGF. They are also involved in the regulation of the epithelial-mesenchymal transition of PCa cells, which contributes to increasing their survival and forming resistance to chemotherapy [15]. According to the recent data, OPN is also involved in the modulation of the tumor microenvironment of some malignant neoplasms, including PCa [16]. However, in the available literature, there is no unified view on the relationship between OPN expression levels and the clinicopathological features associated with the degree of PCa malignancy.

The aim of the work was to study indicators of the OPN expression at the protein and mRNA

levels in PCa cells in order to assess the significance of this protein for predicting the aggressiveness of PCa.

Materials and Methods

Clinical characteristics of patients. The retrospective study is based on the analysis of the results of the examination and treatment of 83 patients with II–IV stage of PCa, who were treated at the State Non-Profit Enterprise "National Cancer Institute" of the Ministry of Health of Ukraine in 2015–2021. All patients gave an informed consent to the use of their clinical data in scientific purposes. All patients underwent clinical, laboratory, and instrumental examinations in accordance with the standards of diagnosis and treatment of patients

Table 1. General clinical characteristics of patients with PCa

Index	Number of patients	
	n	%
Total number of patients	83	100
Age (years)		
Average	62.9 ± 5.8	23
Range	48–76	77
Category T by TNM		
T2	50	60.2
T3	33	39.8
Category N by TNM		
N0	69	83.1
N1	14	12.9
Gleason score		
< 7	20	24.1
≥ 7	63	75.9
Risk of PCa progression by EAU classification		
Low	8	9.6
Moderate	16	19.3
High	59	71.1
Biochemical recurrence		
Established	30	36.1
Not found	53	63.9

with PCa approved by the Order of the Ministry of Health of Ukraine No. 235 of 02.04.2014. The clinical diagnosis was established on the basis of the determination of PSA level in blood serum and digital rectal examination, the results of computer or magnetic resonance imaging, and osteoscintigraphy. The diagnosis of PCa was verified by examining the histological preparations after transrectal multifocal biopsy of the prostate gland under ultrasound control. The stage of the tumor process was determined according to the International Classification of Tumors (TNM, 2009 and 2016). The histological type of resected tumors was verified by morphological examination of histological sections prepared from paraffin blocks (staining with hematoxylin and eosin) in accordance with the International Histological Classification of the WHO (2006). Neoadjuvant therapy was not performed. The detailed clinical characteristics of 83 PCa patients, whose average age was 63.0 ± 5.8 years (with individual range from 45 to 80 years), are given in Table 1. After surgery, patients were examined for 2 years to identify the possible development of biochemical relapse, which was determined when the PSA level increased > 0.2 ng/ml during two consecutive examinations.

Immunohistochemical (IHC) study of the OPN expression in the PCa tissue was performed on 5-µm thick paraffin sections. The monoclonal antibody specific to OPN (clone 441; Thermo Scientific, USA) was used as a primary antibody. To visualize the results of the reaction, a Mouse/Rabbit PolyVue Plus HRP/DAB Detection System reagents kit was used (Diagnostic BioSystems, USA). In accordance with the manufacturer's recommendations, sections were stained with Meyer's hematoxylin (Richard-Allan Scientific, USA). Unmasking of antigens was performed in an EDTA buffer (pH 8.0). The results of IHC studies were analyzed using the H-Score method of counting immunopositive cells on a Primo Star light microscope (Carl Zeiss, Germany) at a magnification of ×400 [17].

The real-time polymerase chain reaction was used to study the expression of SPP1 mRNA in the PCa tissue. Total RNA from the tissue was isolated using the commercial "RNeasy FFPE Kit" (QIAGEN, Germany) according to the manufacturer's protocol. The amount of isolated RNA was determined on a spectrophotometer "NanoDrop 2000c Spectrophotometer" (Thermo Scientific, USA). To study SPP1 mRNA expression, DNA was synthesized from 100 ng of total RNA using the LunaScript® RT SuperMix Kit (New England Biolabs Inc., USA) for reverse transcription. ACTB mRNA was used as an endogenous control to determine mRNA expression. Primer sequences for reverse transcription-PCR (RT-PCR) and real-time PCR were determined using the resource <https://www.ncbi.nlm.nih.gov/tools/primer-blast> and synthesized by Metabion, Germany. The primer sequences were as follows: ACTB forward 5'-GTTACCAACTGGGACGACA-3', reverse 5'-GGGGTGTGTAAGGTCTCAA-3'; SPP1 forward 5'-CGAGGTGATAGTGTGGTTTATGG-3', reverse 5'-GCACCATTCAACTCCTCGCTTTC-3'. The relative expression of the investigated mRNAs was determined by the comparative CT method. The real-time PCR was performed on a QuantStudio 5 Dx Real-Time PCR System (Thermo Scientific, USA). The fold difference between the expression of the studied mRNAs was calculated according to the formula $2^{-\Delta Ct}$ (hereinafter in a.u.). The errors for the fold difference calculations show the range of ΔCt values based on including the standard deviation in these values [18].

Statistical analysis was performed using the software package GraphPad Prism v. 8.00 (GraphPad Software Inc., USA) taking into account the nature of the distribution of the obtained data. Data are presented as median (Me) and the 1st and 3rd quartiles. The Mann — Whitney U-test was used to quantitatively compare two independent groups. The statistical significance of the difference in the expression levels of

the studied markers in three groups was assessed by calculating a Kruskal — Wallis test. Patient survival was analyzed using a Kaplan — Meier method, reliability between curves was analyzed using the log-rank test. For this, all patients were divided into groups with low (\leq Me) and high ($>$ Me) levels of expression of the studied markers. The difference between the values in the groups at $p < 0.05$ was considered statistically significant.

Results

Topology of OPN expression in the PCa tissue. The analysis of the results of the IHC study showed a significant variability of OPN expression in the PCa tissues. A positive reaction with anti-OPN antibody was visualized as brown coloration of varying intensity: from light to dark brown (Fig. 1). The localization of OPN was most often observed in the cytoplasm of tumor cells, less often — in their nuclei. The OPN expression was observed in tumor-infiltrating neutrophils, tumor-associated macrophages, and fibroblasts. It was also noted in the intraluminal secretion of the prostate gland, as well as in *corpora amylacea* and conglomerates, which may be their precursors.

Analysis of OPN expression at the protein and mRNA levels. The study of the quantitative indicators of OPN expression showed that the PCa tissue was characterized by a high level of expression of this protein: Me = 203.0 H-Score points, ranging from 65 to 295 H-Score points. It should be noted that moderate (101—200 H-Score points) and high (201—300 H-Score points) levels of expression of this glycoprotein was found in neoplasms of 65 (78.3%) patients, while low OPN values that did not exceed 100 H-Score points were determined in the tumors of 18 (21.7%) patients.

Next, we analyzed the levels of OPN expression depending on the criteria that have a significant clinical significance for determining the ag-

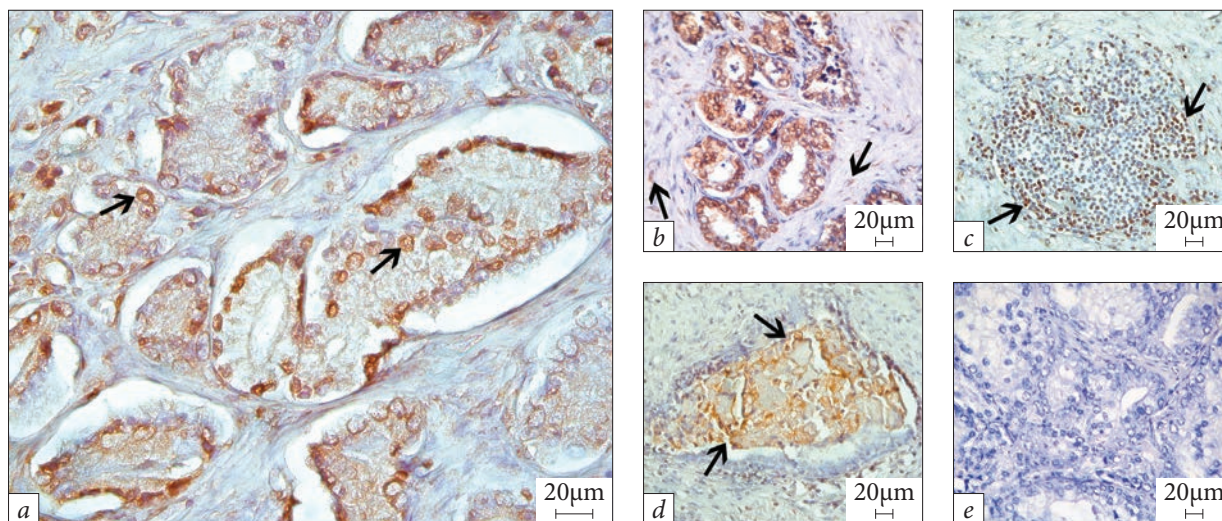


Fig. 1. Expression of OPN in the PCa tissue: (a) in malignantly transformed cells (arrows indicate the localization of OPN in cell nuclei); (b) in stromal cells (marked by arrows); (c) in the cells of the immune infiltrate; (d) in corpora amylacea; (e) negative control. Immunohistochemistry, 3-diaminobenzidine tetrachloride chromogen. Staining with hematoxylin, $\times 260$

gressiveness of the PCa course: the spread of the primary tumor (T by TNM classification), the presence of metastases in regional lymph nodes (N), and the sum of points according to Gleason. As evidenced by the data given in Table 2, OPN expression indicators in the tumor tissue of patients with metastatic lesions of regional lymph nodes were 1.6 times ($p < 0.05$) higher compared to patients without metastases. It was established that the PCa tissues with a Gleason score ≥ 7 were characterized by a 1.4 times ($p < 0.05$) higher OPN expression level compared to tumors with a Gleason score < 7 . No significant difference in OPN expression indicators in the PCa cases of different T categories was detected.

The average value of the mRNA level of *SPP1* in the PCa tissue was equal to $Me = 9.6$ a.u. ranging from 0.2 a.u. to 52.9 a.u. The expression of *SPP1* mRNA was 2.5 times lower in the PCa tissue of patients with metastases in the regional lymph nodes compared to patients without metastases ($p < 0.05$). There was no significant difference in *SPP1* mRNA levels depending on the T category and Gleason score (Table 2).

The relationship of OPN expression levels with the risk of cancer progression and survival of PCa patients. We performed an analysis of OPN expression indicators at the protein and mRNA levels depending on the risk of PCa progression in accordance with the recommendations of the European Association of Urology (EAU) [19]. As can be seen from the data presented in Fig. 2, the tumor tissue of patients with a high risk of PCa progression was characterized by 1.4 and 2.1 times higher OPN levels ($p = 0.0023$) compared to similar indicators of patients with a moderate or low risk of PCa progression, respectively. No significant difference in *SPP1* mRNA expression levels was found depending on the risk of PCa progression (Fig. 2).

At last, an analysis of the 2-year relapse-free survival of patients was performed depending on the OPN expression at the protein and mRNA levels (Fig. 3). It was established that a high level of OPN expression is associated with the significantly lower (by 25.0%; $p = 0.0186$) 2-year relapse-free survival of PCa patients (Fig. 3, a). There was no significant difference in the 2-year

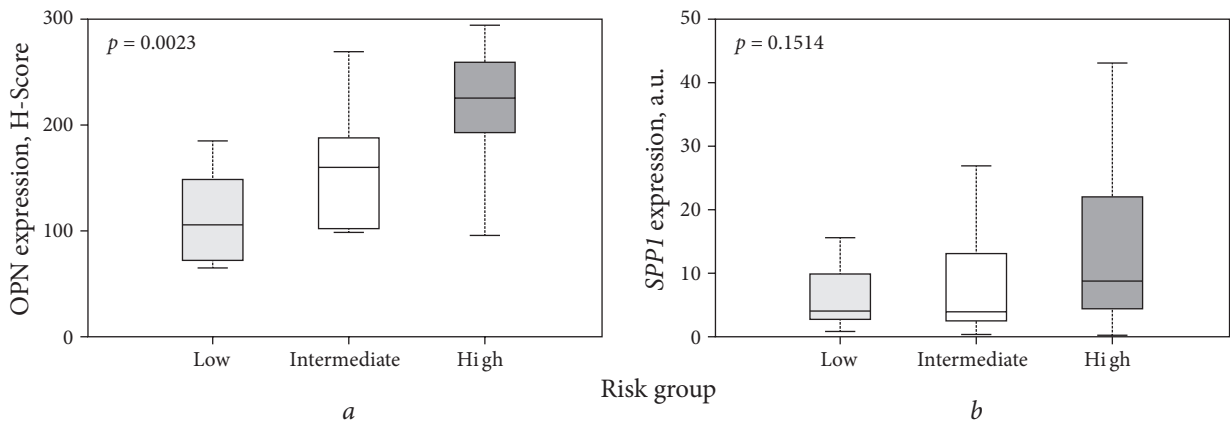


Fig. 2. Dependence of OPN expression indicators at the level of protein (a) and mRNA (b) in the PCa tissue of patients with different risk of cancer progression according to the recommendations of the EAU: in the quartile diagrams (“boxplot”), the central line marks the median, and the lower and upper limits of the “box” indicate the first and third quartiles, respectively. The lines coming out of the rectangles indicate the minimum and maximum value of the indicators in the studied groups

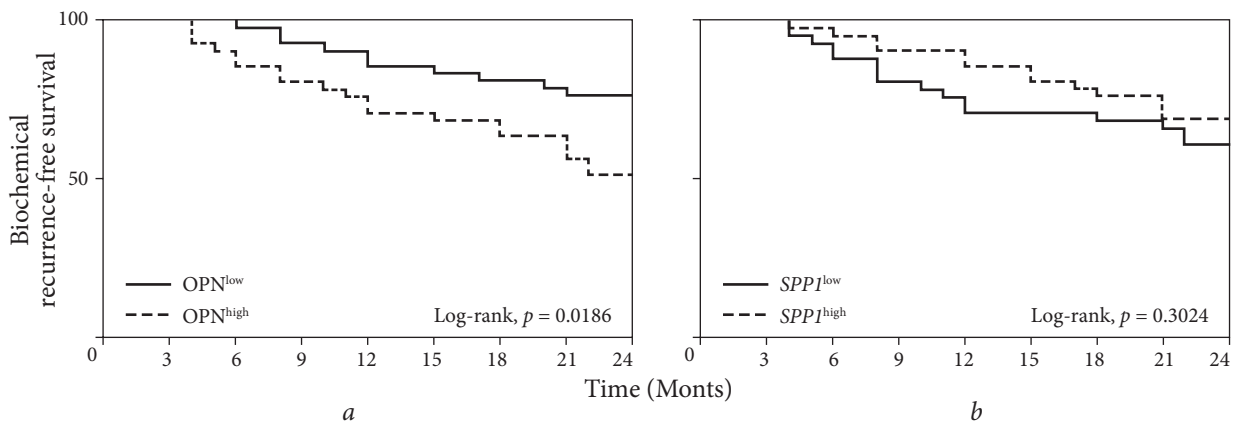


Fig. 3. Relapse-free survival of patients depending on OPN expression indicators at the protein (a) and mRNA levels (b) in the PCa tissue according to the Kaplan — Meier method (log-rank test)

Table 2. Dependence of OPN expression indicators at the protein and mRNA levels on the clinicopathological characteristics of PCa patients

Clinicopathological characteristics		OPN			p	SPP1			p
		Q ₁	Me	Q ₃		Q ₁	Me	Q ₃	
T	T2	150.0	168.5	226.5	0.3203	2.9	8.4	16.8	0.9352
	T3	145.0	245.0	256.0		3.9	6.1	17.3	
N	N0	105.5	157.5	177.3	0.0110	3.7	13.2	23.8	0.0231
	N1	185.0	250.5	266.3		2.6	5.2	11.1	
Gleason score	< 7	72.5	150.0	167.6	0.0202	2.7	4.9	15.6	0.1342
	≥ 7	145.0	215.0	256.0		4.1	9.6	22.4	

recurrence-free survival rate of patients with PCa depending on the *SPP1* mRNA expression in the tumor tissue (Fig. 3, *b*).

Therefore, our results indicate the expediency of further study of the role of OPN in the mechanisms of PCa development and progression to assess the risk of cancer progression.

Discussion

The rapid development of multi-omics technologies and the availability of their results have made it possible to elucidate the peculiarities of tumor cell biology and identify potential diagnostic and prognostic biomarkers of malignant neoplasms, including PCa [20]. Based on the screening of almost 12,000 genes, it was established that OPN is promising for predicting the cancer course of various tumor types [21].

OPN is an acidic glycoprotein with a molecular weight of 44–75 kDa, which belongs to the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family. Today, at least three isoforms of this protein are known: OPNa, OPNb, and OPNc, which differ in their functions and tissue localization [22]. It has been shown that the functional activity of OPNb and OPNa is mediated by integrins, while the action of OPNc is exerted via integrin-independent mechanisms. It is also worth noting that the OPNc isoform is characterized by nuclear localization in the cell, which may be related to its participation in cell division. It is assumed that the mechanisms regulating the translocation of OPN from the cell cytoplasm to the nucleus may be associated with exportin-1-dependent and -independent pathways [23].

It has been established that this glycoprotein is involved in the regulation of cell adhesion, migration, proliferation, survival, differentiation, and immune modulation. Due to its multifunctionality, OPN is involved in various physiological and pathological processes, including wound healing, biomineralization, bone remodeling,

vascularization, diabetes, obesity, inflammation, fibrosis, urolithiasis, autoimmune diseases, and malignancies [24]. According to the literature data [25], the violation of the OPN expression has been determined in 34 types of cancer. Today, OPN is considered a prognostic marker for cancers of the breast, cervix, colorectal, head and neck, liver, lungs, ovaries, etc. It has been established that high levels of OPN in the blood serum of patients are associated with the development and progression of mesothelioma, colon, lung, and breast cancer [26], while an increased OPN expression is determined in the tissue of colorectal cancer, lung cancer, melanoma, breast cancer, gastric cancer, pancreatic cancer, as well as in gliomas [27, 28].

The data we obtained regarding OPN expression both at the mRNA and protein levels indicate its significant prognostic potential in PCa. The identified features of OPN topology in tumor tissue along with the relocalization of its expression in the nuclei of malignantly transformed cells confirm the impairment of the functional activity of this protein during the development and progression of PCa. The relationship between OPN expression levels in the PCa tissue and such clinico-pathological characteristics as the presence of metastatic lesions of regional lymph nodes and the Gleason score was established. It was proven that the level of OPN expression in tumor tissue at the protein level directly correlate with the risk of PCa progression and are associated with the low rates of a 2-year recurrence-free survival. These facts confirm the results of previously published studies on the key role of OPN in the occurrence and progression of PCa and are also consistent with literature data [14].

The relationship between OPN expression and the clinicopathological characteristics of PCa can be explained by the participation of this glycoprotein in the regulation of Akt, Raf/MEK/ERK, and ILK/PI3K/GSK-3 β signaling pathways [29, 30]. It is also worth noting that a high level of OPN expression in human PC-3 cells is asso-

ciated with an increase in the number of invadopodia and gelatinolytic activity, which indicates its role in the modification of structural components to facilitate the invasion of tumor cells through integrin $\alpha v \beta 3$ [31].

Thus, the high expression of OPN at the protein level against a low level of mRNA expression of the *SSP1* gene in tumor tissue is associated with an unfavorable PCa course, which is characterized by a high degree of malignancy according to clinicopathological indicators.

The obtained results indicate the prospects for using OPN expression indicators in tumor tissue

in predicting the aggressiveness of PCa and assessing the risk of its recurrence.

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Н. Лук'янова¹, Т. Задвортний¹, Т. Борікун¹, О. Мушій¹, А. Павлова¹,
А. Тимошенко¹, Е. Стаховський², Ю. Вітрук², В. Чехун¹

¹ Інститут експериментальної патології, онкології і радіобіології
ім. Р.Є. Кавецького НАН України, Київ, Україна

² Державне некомерційне підприємство «Національний інститут раку»
МОЗ України, Київ, Україна

ЗНАЧЕННЯ ОСТЕОПОНТИНУ ДЛЯ ПРОГНОЗУВАННЯ АГРЕСИВНОСТІ ПЕРЕБІГУ РАКУ ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ

Стан питання. Зростання показників захворюваності та смертності хворих на рак передміхурової залози (РПЗ) визначають необхідність удосконалення методів діагностики та лікування цієї онкопатології. Однією з наріжних проблем сучасної онкоурології є ефективне прогнозування перебігу РПЗ та стратифікації

тактики лікування. Перспективним у цьому напрямку вважається використання прогностичних маркерів, які відображають молекулярно-біологічні особливості пухлин. З огляду на важливу роль матрицелюлярних протеїнів у процесах модуляції пухлинного вогнища та метастазуванні гормонозалежних новоутворень, **метою роботи** було дослідити показники експресії остеопонтину (OPN) на рівні білка та мРНК у тканині РПЗ для з'ясування значення цього протеїну для прогнозування агресивності перебігу пухлинного процесу. **Матеріали та методи.** Робота базується на аналізі результатів обстеження та лікування 83 хворих на РПЗ II—IV стадій, які знаходились на лікуванні у Державному некомерційному підприємстві «Національний інститут раку» МОЗ України в період 2015—2021 рр. Ризик прогресії РПЗ визначали у відповідності до рекомендацій Європейської асоціації урологів. Дослідження експресії OPN на рівні мРНК та білка у тканині РПЗ проведено із застосуванням методів полімеразної ланцюгової реакції у реальному часі та імуногістохімії, відповідно. Статистичний аналіз виконано за допомогою GraphPad Prism v. 8.00. **Результати.** Встановлено, що показники експресії OPN у тканині РПЗ були в 1,6 рази ($p < 0,05$) вищими у хворих із метастазами в регіонарні лімфатичні вузли порівняно з пацієнтами без метастазів. У хворих із сумою балів за Глісоном < 7 експресія OPN в пухлинній тканині була в 1,4 рази меншою ($p < 0,05$), ніж у хворих із низькодиференційованим РПЗ. У пацієнтів із високим ризиком прогресії пухлинного процесу рівень експресії OPN був у 1,4 та 2,1 рази більшим ($p < 0,05$) порівняно з аналогічними показниками хворих із помірним та низьким ризиком прогресії РПЗ. Достовірне зниження показників дворічної безрецидивної виживаності на 25% встановлено у хворих з високим рівнем експресії OPN у тканині РПЗ. **Висновки.** Отримані результати свідчать про доцільність використання показників експресії OPN у пухлинній тканині для прогнозування агресивності перебігу пухлинного процесу та оцінки ризику виникнення рецидиву РПЗ.

Ключові слова: рак передміхурової залози, остеопонтин, прогноз перебігу, виживаність.